Propionibacterium acnes Activates the IGF-1/IGF-1R System in the Epidermis and Induces Keratinocyte Proliferation

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Propionibacterium acnes has a major role in the development of acne lesions. IGF-1 stimulates the proliferation of keratinocytes via an activation of the IGF-1 receptor (IGF-1R). Zinc has been proven to work efficiently against inflammatory acne and to modulate the IGF-1 system. Our objectives were to study the modulation of IGF-1 and IGF-1R expression by *P. acnes* extracts and to determine their modulation by zinc gluconate. *In vivo*, we analyzed biopsies of acne lesions and healthy skin, and *in vitro* we used skin explants incubated with two *P. acnes* extracts—membrane fraction (MF) and cytosolic proteins—with or without zinc. IGF-1 and IGF-1R expression was evaluated using immunohistochemistry, and the IGF-1 production in supernatants was measured by ELISA. Then, IGF-1 and IGF-1R mRNA levels were analyzed using quantitative PCR on normal human epidermal keratinocytes (NHEKs). IGF-1 and IGF-1R were overexpressed in acne lesions. MF increased IGF-1 and IGF-1R expression in the epidermis of explants and was associated with an overexpression of both Ki-67 and filaggrin. Zinc had the effect of downregulating IGF-1 and IGF-1R levels. These observations were confirmed at the mRNA level for IGF-1R in NHEKs. These results demonstrate that *P. acnes* can induce the formation of comedones by stimulating the IGF/IGF-1R system. Moreover, zinc downregulates this pathway.

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INTRODUCTION

Acne vulgaris is a common chronic inflammatory skin disease that affects the pilosebaceous follicle. Three main factors have been identified in the pathogenesis of acne. First, there is hyperproliferation and abnormal differentiation of follicular epithelium keratinocytes. Second, an excessive amount of sebum is produced by the sebaceous glands. Finally, there is inflammation induced by *Propionibacterium acnes* (Eichenfield and Leyden, 1991; Thiboutot, 1997; Zouboulis *et al.*, 2005).

P. acnes is a bacterium that not only induces an inflammatory reaction but also maintains it. By releasing lipases, *P. acnes* induces production of free fatty acids. It also secretes chemotactic factors (Vowels *et al.*, 1995; Nagy *et al.*, 2005; Schaller *et al.*, 2005) and increases the secretion of proinflammatory cytokines (tumor necrosis factor-α, IL-1β, and IL-8) from mononuclear cells and keratinocytes (Vowels *et al.*, 1995; Sugisaki *et al.*, 2009). *P. acnes* also induces the activation of Toll-like receptors-2 and -4 in keratinocytes (Jugeau *et al.*, 2005; Nagy *et al.*, 2006). Furthermore, the *P. acnes* genome encodes many factors that may have inflammatory potential (Bruggemann *et al.*, 2004). It was recently reported that this bacterium also modulates keratinocyte proliferation and differentiation through an induction of filaggrin and integrin expression (Jarrousse *et al.*, 2007a), but the mechanism remains unknown.

There is increasing evidence suggesting the involvement of IGF-1 in acne. Recent studies describe a correlation between IGF-1 serum levels and the severity of acne in women (Aizawa and Niimura, 1995; Cappel *et al.*, 2005). IGF-1 serum levels also correlate directly with the amount of facial sebum in both men and women (Vora *et al.*, 2008). In the skin, IGF-1 induces keratinocyte proliferation *in vitro* (Ristow and Messmer, 1988; Krane *et al.*, 1991; Barreca *et al.*, 1992) and *in vivo* (Gilhar *et al.*, 1994; Valentinis and Baserga, 2001; Sadagurski *et al.*, 2006), and it induces lipid production in human sebocytes (Smith *et al.*, 2006). In addition, inhibition of IGF-1 receptor (IGF-1R) results in a thin abnormal epidermis (Liu *et al.*, 1993).

Several studies have reported beneficial effects of zinc salts on inflammatory lesions in mild to moderate acne (Dreno *et al.*, 1989; Michaelsson and Ljunghall, 1990;

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Abbreviations: CP, cytosolic protein; Ctrl, control; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IGF-1R, IGF receptor 1R; IGFBP-3, IGF-binding protein-3; LPS, lipopolysaccharide; MF, membrane fraction; NHEK, normal human epidermal keratinocyte; P. acnes, Propionibacterium acnes

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Schachner *et al.*, 1990; Meynadier, 2000). A link between zinc and the IGF-1 system has recently been reported in the downregulation of IGF-1R expression in prostate cancer cells (Banudevi *et al.*, 2010).

In this context, the aim of our work was first to study the *in vivo* IGF-1 and IGF-1R expression levels in the epidermis of inflammatory acne lesions as compared with healthy skin. Second, we investigated whether *P. acnes* was able to induce IGF-1R expression and IGF-1 secretion by keratinocytes. Finally, we evaluated the modulation of IGF-1 or IGF-1R by zinc gluconate.

RESULTS

In vivo expression of IGF-1, IGF-1R, Ki-67, and filaggrin in acne lesions and healthy skin

We analyzed three acne biopsies from the upper back (inflammatory papules) and three healthy skin biopsies from the same region. In all the acne biopsies, we observed a strong expression of IGF-1 (Figure 1b) and IGF-1R (Figure 1d) in the epidermis as compared with healthy skin (Figure 1a and c). In addition, Ki-67 expression was strongly increased in the basal layer of the epidermis of acne lesions (Figure 1f) as compared with healthy skin (Figure 1e). In comparison with healthy skin (Figure 1g), in which filaggrin expression is located only in the stratum corneum, among the acne lesions (Figure 1h), we observed expression in the stratum corneum and the suprabasal layers of the epidermis.

In vitro modulation of IGF-1, IGF-1R, Ki-67, and filaggrin expression by *P. acnes* extracts

IGF-1 and IGF-1R modulation by* P. acnes *extracts in cutaneous explants. After we confirmed that expression of IGF-1 and IGF-1R was similar both in healthy abdominal and in healthy upper-back skin (data not shown), we used abdominal skin explants to evaluate the effects induced by *P. acnes* (Figures 2–4). After 3 hours, induction of IGF-1 expression in the epidermis was noted with both *P. acnes* membrane fraction (MF) and lipopolysaccharide (LPS), but it was not statistically significant as compared with the control (Ctrl) medium ($P>0.05$; Figure 2a and b). IGF-1R was significantly increased, up to 4.00 ± 0.89 with *P. acnes* MF, as compared with the control medium 1.50 ± 0.55 ($P<0.05$).

After 6 hours, IGF-1 expression was significantly increased, from 1.67 ± 0.52 in the Ctrl medium to 4.00 ± 0.89 with *P. acnes* MF only ($P<0.05$). IGF-1R expression

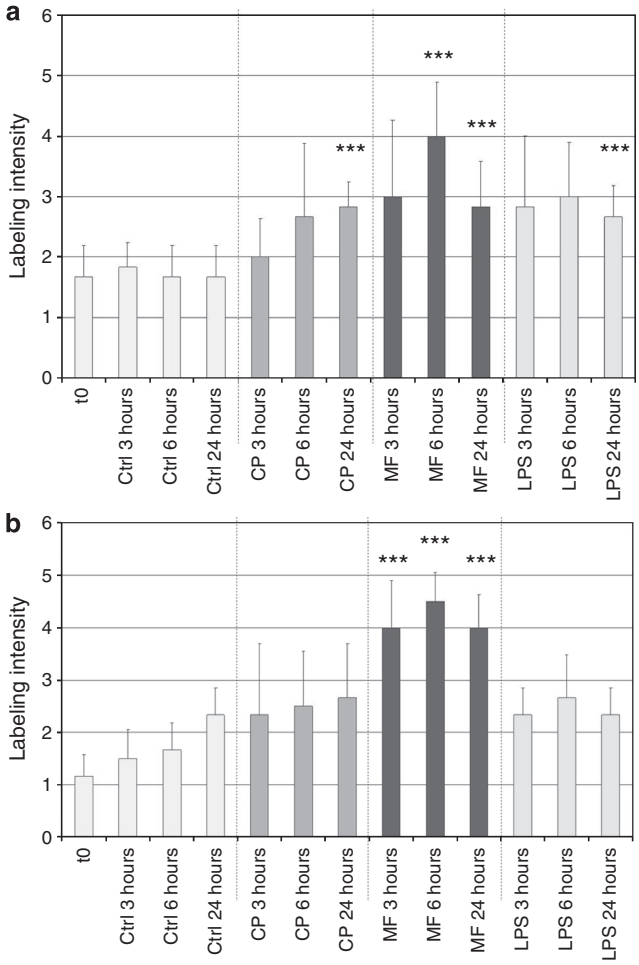

Figure 2. *P. acnes* modulates IGF-1 and IGF-1R expression. Expression of IGF-1 (**a**) and IGF-1R (**b**) in the epidermis of explants without or after 3, 6, or 24 hours of incubation with *P. acnes* membrane fraction (MF), cytosolic protein (CP), and lipopolysaccharide (LPS). Scale: null labeling (0), very weak labeling (1), weak labeling (2), moderate labeling (3), strong labeling (4), and very strong labeling (5). ***Significant modulation. Ctrl, control.

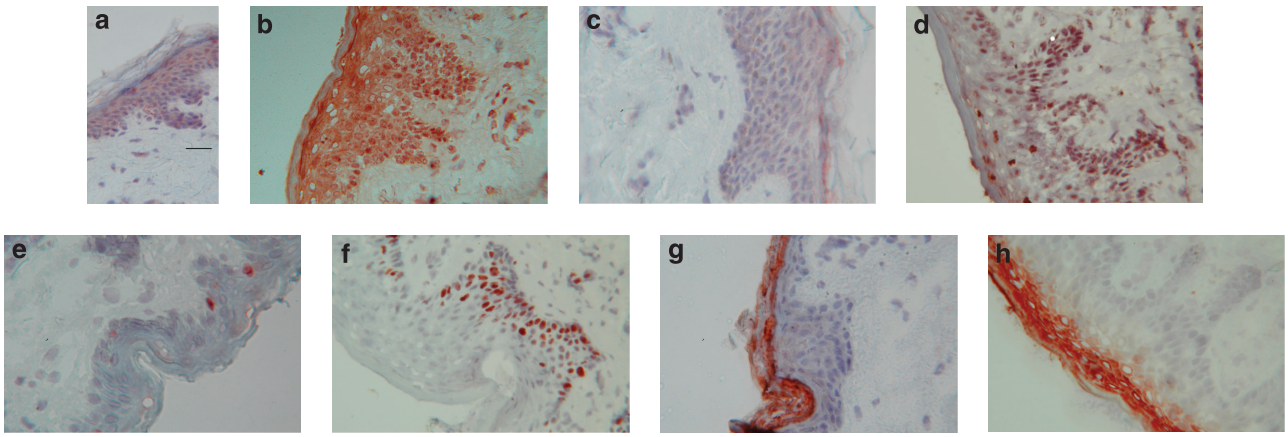

Figure 1. Cutaneous expression of IGF-1, IGF-1R, Ki-67, and filaggrin. Expression of IGF-1 (**a** and **b**), IGF-1R (**c** and **d**), Ki-67 (**e** and **f**), and filaggrin (**g** and **h**) in the epidermis of healthy donors (**a**, **c**, **e**, and **g**) and acne lesions (**b**, **d**, **f,** and **h**). Bar = 30 μm.

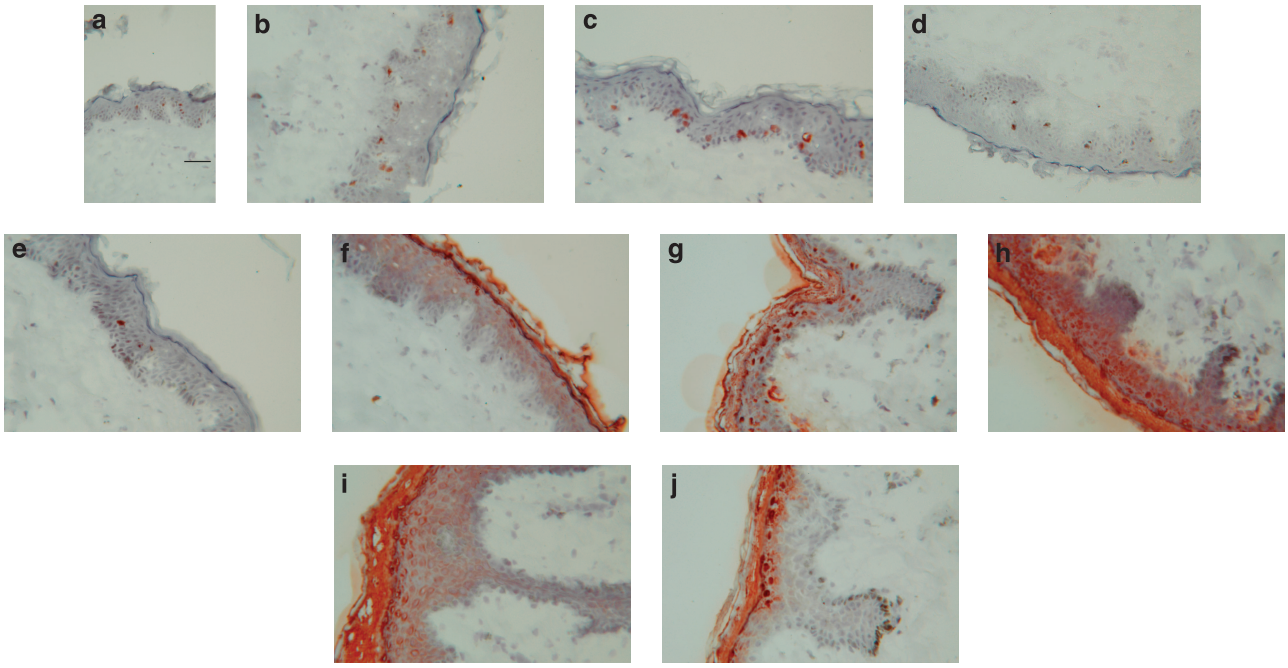

Figure 3. Ki-67 and filaggrin expressions are modulated by *P. acnes* and zinc gluconate. Detection of Ki-67 (upper slides) and filaggrin (lower slides) expression in healthy abdominal skin (**a** and **f**), after 6 hours of incubation with lipopolysaccharide (**b** and **g**) or *P. acnes* membrane fraction (**c** and **h**), and with a preincubation of 1 hour (**d** and **i**) or 24 hours (**e** and **j**) with zinc gluconate alone before membrane fraction addition for 6 hours. Bar = 50 μm.

significantly increased, from 1.67 ± 0.52 to 4.50 ± 0.55 with *P. acnes* MF ($P<0.05$).

After 24 hours, IGF-1 expression was significantly increased, from 1.67 ± 0.52 in the Ctrl medium to 2.83 ± 0.75 with *P. acnes* MF (<0.05), 2.83 ± 0.41 with *P. acnes* cytosolic protein (CP) ($P<0.05$), and 2.67 ± 0.52 with LPS ($P<0.05$). IGF-1R expression was also increased, from 2.33 ± 0.52 to 4.00 ± 0.63 with *P. acnes* MF only (<0.05).

Ki-67 and filaggrin modulation by* P. acnes *extracts in cutaneous explants. Ki-67 expression was strongly increased in the basal layer of the epidermis after 6 hours incubation with *P. acnes* MF (Figure 3c), less with LPS (Figure 3b) as compared with the Ctrl medium (Figure 3a). *P. acnes* MF (Figure 3h), and, less intensively, LPS (Figure 3g) extended the expression of filaggrin from the stratum corneum to the lower part of the epidermis as compared with the control medium (Figure 3f).

IGF-1 ELISA after 6 hours of stimulation with* P. acnes *in cutaneous explant supernatants. After 6 hours, IGF-1 secretion was increased to 0.34 ± 0.13 ng l^{-1} with *P. acnes* MF, 0.28 ± 0.08 ng l^{-1} with *P. acnes* CP, and 0.25l ± 0.05 ng l^{-1} with LPS as compared with the control medium, 0.23 ± 0.10 ng l^{-1} (Figure 4a). Although the modulation noted was not significant, it was nonetheless observed for all six donors studied.

IGF-1 and IGF-1R quantitative reverse transcription–PCR analysis after 6 hours of stimulation with* P. acnes *on normal human epidermal keratinocytes. After 6 hours stimulation with *P. acnes* MF, IGF-1R mRNA level was significantly increased as compared with the control medium, with an induction factor of 1.03 ± 0.14 ($P=0.02$; Figure 4b). A significant reduction of IGF-1R mRNA level was noted after 6 hours stimulation with LPS (inhibition factor = 0.86 ± 0.02; $P=0.0001$). IGF-1 mRNA was not detected in normal human epidermal keratinocytes (NHEKs).

***In vitro* modulation of keratinocyte IGF-1, IGF-1R, Ki-67, and filaggrin expression by zinc salts**

IGF-1 and IGF-1R modulation in cutaneous explants. Before addition of MF for 6 hours, a 1-hour preincubation with zinc gluconate partially inhibited IGF-1 overexpression induced by MF, but the effect was significant only after 6 hours (4.00 ± 0.89 to 2.33 ± 0.52; $P<0.05$; Figures 3–5). In a similar manner, the expression of IGF-1R induced by MF (1, 3, and 6 hours) was significantly inhibited by a 1-hour preincubation with zinc ($P<0.05$; Figure 5a and b).

A 24-hour preincubation with zinc gluconate resulted in a strong downregulation of IGF-1 expression induced by MF (1.00 ± 0.00 after 3 hours, 1.33 ± 0.52 after 6 hours, and 1.17 ± 0.41 after 24 hours), leading to a recovery of IGF-1 initial levels ($P<0.05$). The same results were obtained with IGF-1R, leading to a recovery of initial IGF-1R levels ($P<0.05$).

Ki-67 and filaggrin modulation with zinc preincubation in cutaneous explants. A 1-hour preincubation with zinc gluconate, followed by 6 hours of incubation with MF, inhibited the induction of both Ki-67 (Figure 3d) and filaggrin (Figure 3i) expression by MF alone (Figure 3c and h). This inhibition was more pronounced with 24 hours of preincubation, leading to a recovery of the initial levels of Ki-67 (Figure 3e) and filaggrin expression (Figure 3j).

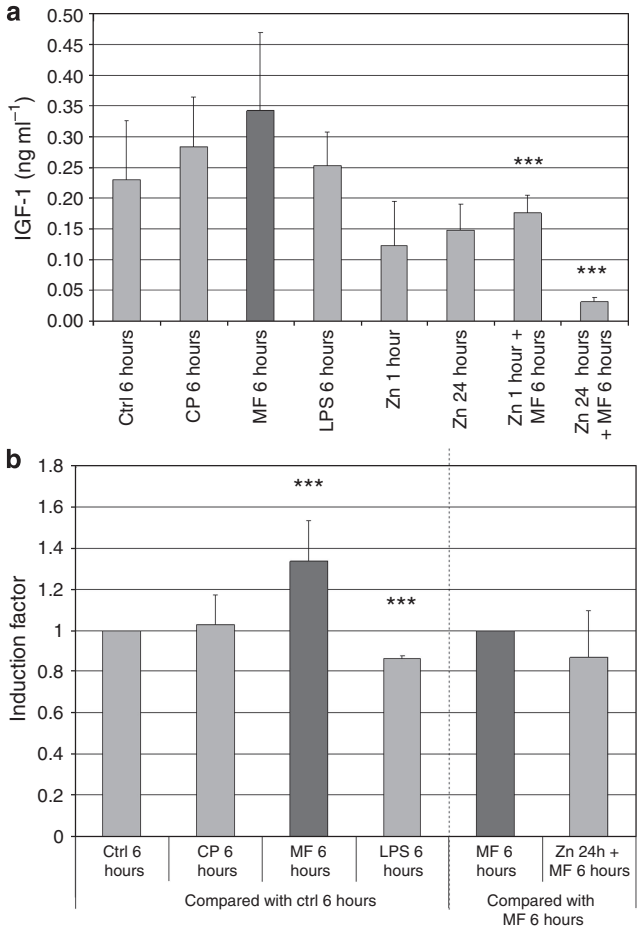

Figure 4. IGF-1 and IGF-1R expression is stimulated by *P. acnes* and modulated by zinc gluconate. (**a**) IGF-1 levels in explant culture supernatants. Secretion of IGF-1 in explant culture supernatants after 6 hours of incubation with or without *P. acnes* membrane fraction (MF), cytosolic protein (CP), and lipopolysaccharide (LPS) alone, or with a preincubation of 1 or 24 hours with zinc gluconate before MF addition. Zinc alone for 1 h or 24 hours was used as a control (Ctrl). ***Significant modulation. (**b**) IGF-1R mRNA levels in normal human epidermal keratinocytes. *Left*: IGF-1R mRNA relative expression after 6 hours incubation with or without *P. acnes* MF, CP, and LPS alone as compared with the basal level (Ctrl), which was regarded as 1. *Right*: IGF-1R mRNA relative expression after 24 hours with zinc gluconate before MF addition for 6 hours as compared with the MF-induced level (MF 6 hour), which was regarded as 1. ***Significant modulation.

ELISA of zinc salt modulation of keratinocyte IGF-1 expression. A 1-h preincubation with zinc gluconate significantly reduced the IGF-1 overexpression induced by MF, from 0.34 ± 0.13 to 0.18 ± 0.03 ng ml^{-1} ($P<0.05$; Figure 4a).

This reduction was more pronounced after 24-hours preincubation with zinc gluconate (0.34 ± 0.13 to 0.03 ± 0.01 ng ml^{-1}; $P<0.05$).

Quantitative real-time RT-PCR analysis of zinc salt modulation of IGF-1R in NHEKs. Preincubation with zinc for 24 hours reduced the IGF-1R mRNA expression induced by MF (6 hours stimulation), with an inhibition factor of 0.86 ± 0.23 ($P=0.66$; Figure 4b). Although the modulation observed was not significant, it was noted for four of the five donors studied.

DISCUSSION

In this study, we found that IGF-1 and IGF-1R are overexpressed in acne lesions as compared with healthy skin. To our knowledge it has not previously been reported that *P. acnes* stimulates IGF-1 and IGF-1R expression in keratinocytes and increases IGF-1 secretion. In addition, we observed that zinc gluconate prevents the induction of both IGF-1 and IGF-1R expression by *P. acnes* in keratinocytes. Furthermore, IGF-1 and IGF-1R overexpression is associated with an increase in the proliferation index of Ki67 and filaggrin expression. These two markers were also downregulated by zinc gluconate. Of the two extracts of *P. acnes*, MF has the strongest stimulating effect on IGF-1 and IGF-1R expression. Although MF contains membrane and cell-wall components, particularly peptidoglycan and lipoteichoic acid, CP contains only cytosolic proteins. Thus, these results suggest that *P. acnes* acts on the IGF-1/IGF-1R system through a direct contact with the keratinocyte membrane, which could be lipoteichoic acid, a major cell-wall component of Gram-positive bacteria, previously found to increase cell proliferation among human ureteral epithelial cells (Wille *et al.*, 1992; Elgavish *et al.*, 1996). In addition, the induction of IGF-1 and IGF-1R expression was always more pronounced than with LPS, which is considered a reference among proinflammatory substances. However, LPS shows an inhibitory effect on IGF-1R mRNA expression in NHEKs but not in explants, suggesting its specific role in keratinocytes of the basal layer.

Interestingly, we observed that IGF-1 and IGF-1R overexpression in both acne lesions and skin explants was associated with an increase in Ki-67 and filaggrin expression in the epidermis, confirming that the IGF-1/IGF-1R system is associated with the modulation of both proliferation and differentiation of keratinocytes. It was previously noted that Ki-67 expression is higher in acne patients' epidermis than in normal healthy skin (Knaggs *et al.*, 1994). Moreover, we have reported that the differentiation of keratinocytes is modulated by *P. acnes* in the epidermis through an induction of filaggrin and integrin expression (Jarrousse *et al.*, 2007a). Taken together, these results argue for the hypothesis that *P. acnes* has a significant role in the formation of comedones and that one of the mechanisms implicates the IGF-1/IGF-1R pathway (Figure 6).

Recently, acne has been suggested to be an IGF-1-mediated disease, modified by diets that increase IGF-1 signaling (Melnik and Schmitz, 2009). A direct relationship between consumption of milk and other dairy products and elevated IGF-1 serum levels has been established (Crowe *et al.*, 2009; Qin *et al.*, 2009). Although to date no clear association has been established between acne prevalence and diet (Davidovici and Wolf, 2010; Melnik, 2010), recent studies describe a correlation between milk consumption and acne prevalence (Spencer *et al.*, 2009). Thus, IGF-1 signaling may have a major role in the formation of acne lesions. Given that IGF-1 has a higher affinity for IGF-1R than does IGF-2 or insulin (Hodak *et al.*, 1996), we can hypothesize that the

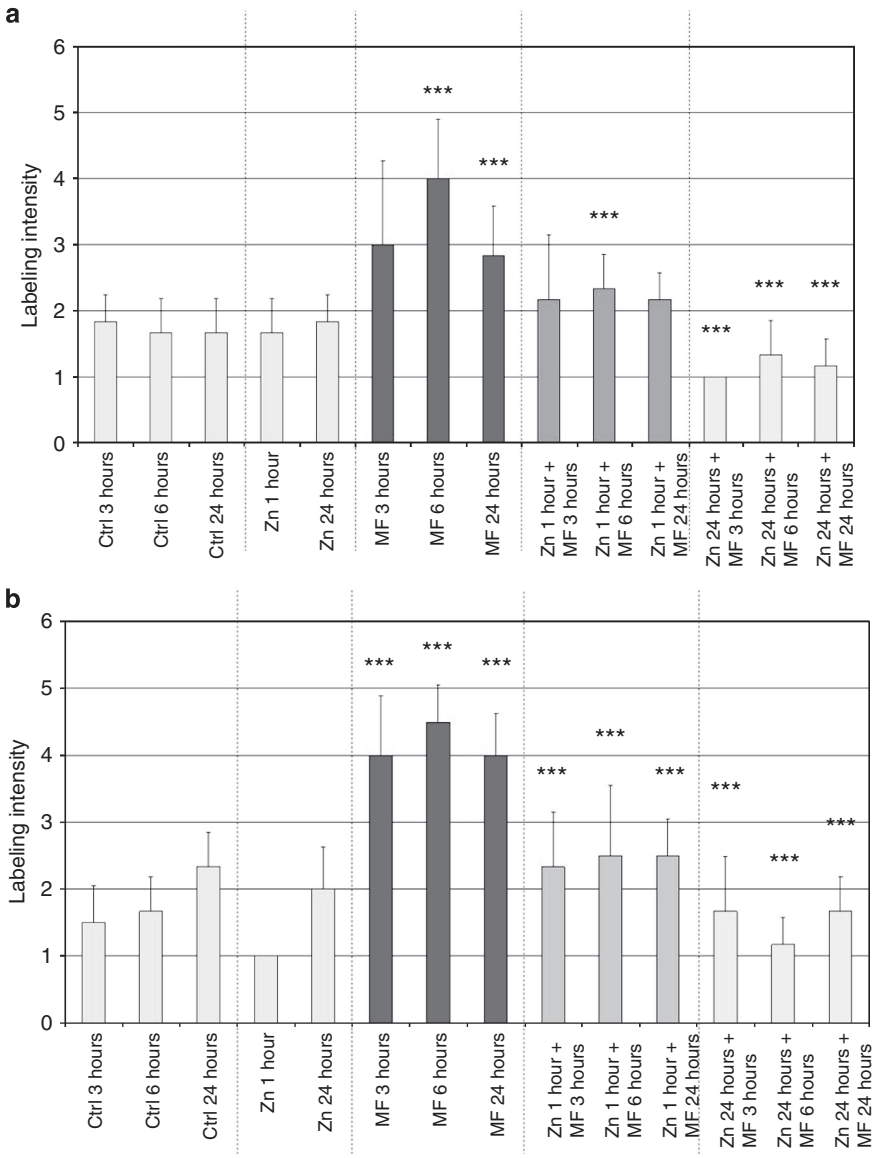

Figure 5. Zinc gluconate downregulates IGF-1 and IGF-1R expression. Expression of IGF-1 (**a**) and IGF-1R (**b**) in the epidermis of healthy skin, after 3, 6, and 24 hours of incubation with *P. acnes* membrane fraction (MF) alone or after a preincubation of 1 or 24 hours with zinc gluconate before MF addition. Zinc alone for 1 or 24 hours was used as a control (Ctrl). Scale: null labeling (0), very weak labeling (1), weak labeling (2), moderate labeling (3), strong labeling (4), and very strong labeling (5). ***Significant modulation.

IGF-1/IGF-1R pathway has a central role in comedogenesis and is activated by two mechanisms. First, a high glycemic diet has been shown to increase IGF-1 and insulin serum levels. Second, our results show that *P. acnes* alone is able to activate the keratinocyte IGF-1/IGF-1R system. These two mechanisms may act synergistically. The absence of IGF-1 mRNA in NHEKs confirms that IGF-1 is produced mainly by keratinocytes of the suprabasal layers (Rudman *et al.*, 1997; Tavakkol *et al.*, 1999). This IGF-1 production can activate IGF-1R, mostly located in the basal layer of epidermis, and thus induce proliferation of keratinocytes and an increase in filaggrin expression through a paracrine pathway. Moreover, acne lesion formation is increased by the induction of lipogenesis (Smith *et al.*, 2008) and the production of androgens (Melnik, 2009) by IGF-1 (Figure 6). Thus, these results establish a link among hyperglycemic diets, *P. acnes,* and acne lesions.

With respect to zinc, it has been shown that this essential trace element has mainly anti-inflammatory properties; it induces inhibition of polymorphonuclear leukocyte chemotaxis (Dreno *et al.*, 1992), decreases nitric oxide production (Yamaoka *et al.*, 2000), induces inhibition of tumor necrosis factor-α and IL-6, and decreases Toll-like receptor-2 expression (Jarrousse *et al.*, 2007b). In addition, zinc is known to have an antimicrobial activity against *P. acnes* (Fluhr *et al.*, 1999; Dreno *et al.*, 2005).

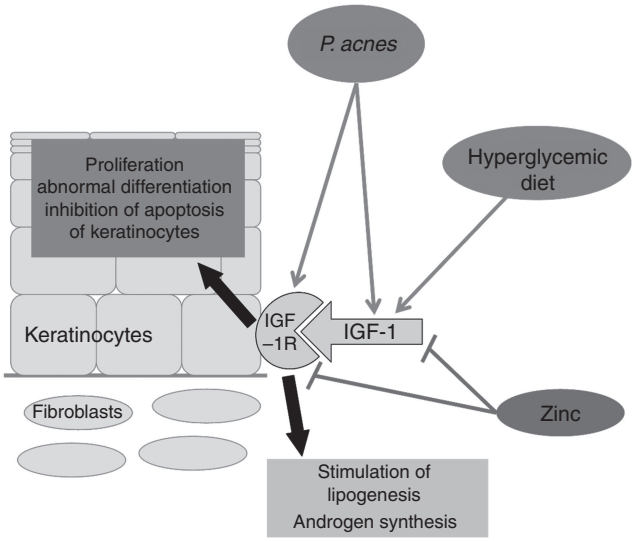

Figure 6. IGF-1/IGF-1R system in acne lesion development.

In this study, we demonstrated that zinc prevents the activation of IGF-1 and IGF-1R expression induced by *P. acnes*. Because zinc is known for being able to modify protein conformation (Stray *et al.*, 2004), it could act by modulating some proteins implicated in the transcription or translation of the *IGF-1* or *IGF-1R* genes. This effect was obtained with as little as 1 hour of preincubation with zinc and was complete after 24 hours of preincubation. Interestingly, this IGF-1/IGF-1R system inhibition is associated with a preventive effect on both hyperproliferation and abnormal differentiation of the epidermis.

Very recently, in a similar manner, it has been shown that isotretinoin was able to modulate the IGF system. Indeed, isotretinoin—the most efficient treatment against acne—has been observed to decrease both serum IGF-1 and IGF-binding protein-3 (IGFBP-3) levels (Karadag *et al.*, 2010). IGFBP-3 is the most abundant IGFBP in plasma, has the highest affinity for IGF-1, and modulates keratinocyte differentiation (Grimberg and Cohen, 2000; Edmondson *et al.*, 2005). Thus, IGFBP-3 is strongly implicated in the IGF system, and it would be of interest to examine its modulation by zinc (Singh *et al.*, 2004). Finally, a recent study found that external stressors such as the bacteria *Mycobacterium leprae* are implicated in the induction of the keratinocyte IGF-1/IGF-1R signaling with induction of cell proliferation (Rodrigues *et al.*, 2010). Taken together, these results support a major role for the IGF-1/IGF-1R system in acne pathology, as early as during the formation of the microcomedo.

In conclusion, IGF-1 and IGF-1R have been identified as targets for *P. acnes* in the development of acne lesions. We have confirmed that *P. acnes* acts not only in the inflammatory step of acne pathogenesis but also in the retentional stage. This probably explains why both antibiotics and benzoyl peroxide have a moderate antiretentional effect by killing the bacteria. Finally, zinc, like isotretinoin, modulates the IGF/IGF-1R system.

MATERIALS AND METHODS

Materials

Bacterial extracts. P. acnes *extracts*: Two *P. acnes* IP53113T (Pierre Fabre, Toulouse, France) extracts were made available to us. The strain was first described in 1968. The MF of the bacteria contains peptidoglycan and teichoic acid. The second fraction was the CP fraction of the bacteria. The MF of the bacteria was resuspended in culture medium.

LPS used as proinflammatory substance: The LPS extracted from *Escherichia coli 0111:B4* (Sigma-Aldrich, St Quentin Fallavier, France) was reconstituted in phosphate-buffered saline and used as a positive control for inflammation. The LPS was diluted in Dulbecco's modified Eagle medium (Sigma-Aldrich) with a final concentration of 1 μg ml^{-1} and incubated with cutaneous explants.

Trace element: zinc salts. Zinc gluconate (Labcatal, Montrouge, France; 15 mg zinc gluconate = 2 mg zinc element) was diluted in Dulbecco's modified Eagle medium with a final zinc concentration of 1 μg ml^{-1} and incubated with cutaneous explants.

In vitro *study: healthy skin explants and NHEKs.* Normal human cutaneous explants—an *in vitro* condition modeling the *in vivo* situation—were obtained from surgical samples of healthy abdominal skin from the Plastic Surgery Service of the Nantes University Hospital (France).

NHEKs were obtained from foreskins of healthy donors at Nantes University Hospital (France). Five donors were used for the quantitative reverse transcription–PCR analysis.

In vivo *study: skin biopsies.* Three cutaneous biopsies of inflammatory acne lesions (papules) of the upper back and three biopsies of healthy skin from the same area were obtained from a skin tissue bank (Department of Dermato-Oncology, Nantes University Hospital, France). All patients provided informed consent. The study was conducted according to the principles of the Declaration of Helsinki, and the Medical Ethical Committee of the Nantes University Hospital approved all described studies.

Methods

Skin explant technique. Punches (4 mm in diameter) from the abdominal skin of six healthy donors, considered a healthy skin model, were incubated at 37 °C in a moist atmosphere in the presence of 5% CO_2 for 3, 6, or 24 hours in Dulbecco's modified Eagle medium as described in a previous study (Jarrousse *et al.*, 2007a). The medium contained *P. acnes* extracts at the following concentrations: MF 1/2 or CP 1/5, LPS at 1 μg ml^{-1} or zinc at 1 μg ml^{-1}. The medium alone was used as a control. After incubations of 3, 6, or 24 hours, the explants were removed from the culture medium and frozen at −80 °C. In order to evaluate any zinc effects, the explants were preincubated with zinc at 1 μg ml^{-1} for 1 hour or 24 hours and then incubated for 6 hours with MF with a final zinc concentration of 1 μg ml^{-1} and MF 1/2.

NHEK culture. NHEKs obtained from healthy foreskins were grown at 37 °C in a humid environment under 5% CO_2 in keratinocyte serum-free medium (Invitrogen, Cergy-Pontoise, France) supplemented with 5 ng ml^{-1} EGF, 25 μg ml^{-1} bovine pituitary extract, 2.5 μg ml^{-1} Fungizone (Bristol-Myers Squibb, Rueil Malmaison, France), and 1% penicillin/

streptomycin (Sigma-Aldrich). Cells were used after a limited number ($n=2$) of subcultures and were incubated with MF, CP, LPS, or zinc. The medium alone was used as a control. Cells were preincubated with zinc for 24 hours and then incubated for 6 hours with MF at a final concentration of zinc at 1 μg ml^{-1} and MF 1/2.

Immunoperoxidase. Immunohistochemistry was performed using the streptavidin/peroxidase technique as previously described (Jarrousse *et al.*, 2007a). We used frozen sections (5 μm thick) of cutaneous biopsies of acne lesions, normal skin, or explants. Primary antibodies were deposited on the slides for 1 hour for IGF-1 (R&D Systems, Lille, France) and IGF-1R (R&D Systems) or 30 minutes for Ki67 (Dako, Trappes, France) and filaggrin (Biomedical Technologies, Cliniscience, Montrouge, France) in a humid environment at room temperature. Two examiners read the slides. Labeling intensity was scored on a five-point scale: null labeling (0), very weak labeling (1), weak labeling (2), moderate labeling (3), strong labeling (4), and very strong labeling (5).

Microscopy and scoring. Slides were read using a Leica microscope (Leica Microsystems, Nanterre, France), and photographs were taken with a digital SLR Camera D70S (Nikon, Champigny Sur Marne, France). All exposures were for the same length of time, at the same luminosity, and at the same magnification ($\times 25$). The immunostaining intensity was scored by two independent observers, who were unaware of the tissue source or incubation conditions.

ELISA. IGF-1 concentrations in cutaneous explant culture supernatants were measured by ELISA using the commercial Human IGF-1 Quantikine Kit (R&D Systems), according to the manufacturer's protocol. All culture supernatants were collected and concentrated using the Amicon Ultra-4 Membrane Ultracell-3 (Millipore, Molsheim, France).

Quantitative real-time reverse-transcription PCR. NHEKs were lysed in RLT buffer (Qiagen, Courtaboeuf, France) supplemented with 0.1% β-mercaptoethanol (Sigma). mRNA extraction was performed using the RNeasy Mini Kit (Qiagen). mRNA concentrations were evaluated using Nanodrop (Thermo Scientific, Illkirch, France) and RNA integrity using Agilent technology (Villecresnes, France). Reverse transcription was performed using 2 μg of total RNA and relative quantification of *IGF-1, IGF-1R,* and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) expression was performed using real-time PCR as previously described (Pfaffl, 2001), with the corresponding TaqMan probes (TaqMan gene expression assays; Applied Biosystems, Courtaboeuf, France) and 20 ng of complementary DNA samples, in an Mx3005P machine (Stratagene, Lyon, France). Thermal cycling was at 50 °C for 2 minutes and 95 °C for 10 minutes, followed by 45 cycles at 95 °C for 30 seconds and 60 °C for 1 minute. PCR efficiency was determined using Total Liver RNA (Biochain, Montrouge, France), performed in parallel to plot the standard curves for *IGF-1, IGF-1R,* and *HPRT.* Mean threshold cycle (CT) values from duplicate PCRs were normalized to mean CT values for the housekeeping gene (*HPRT*) from the same complementary DNA preparations. The relative expression ratio of a target gene was calculated on the basis of the PCR efficiency (E) and the CT deviation between a given NHEK sample (x) and nonstimulated NHEK samples (calibrator), expressed in comparison with *HPRT:* ratio $= (E\ IGF\text{-}1R)^{\Delta CT\ IGF-1R\ (calibrator\_x)} / ((E\ HPRT)^{\Delta CT\ HPRT(calibrator\_x)})$.

Statistical analysis. IGF-1 and IGF-1R protein levels were expressed as the mean ± SD. To determine the differences between all experimental groups (Ctrl, CP, MF, LPS, zinc), a Kruskal–Wallis test was performed. If the test was significant, a Dunnett test was used to isolate the group(s) that differed from the Ctrl group. This closed test procedure allows the alpha risk to remain at 5%. For purposes of the IGF-1R mRNA analysis, mRNA relative expressions were expressed as the mean ± SD compared with the basal level (Ctrl) or the MF-induced level (MF 6 hours).

Confidence intervals (95%) were calculated for each ratio (observed/theoretical) and for each condition. The ratio was considered significant if the 95% confidence interval excluded 1. The significance of the stimulating effects of CP, MF, LPS, or zinc as compared with controls and the inhibiting effect of zinc preincubation, as compared with MF alone, were all assessed at the $P<0.05$ value (***).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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